Requirement of Nucleotide Exchange Factor for Ypt1 GTPase Mediated Protein Transport

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Abstract. Small GTPases of the rab family are involved in the regulation of vesicular transport. It is believed that cycling between the GTP- and GDP-bound forms, and accessory factors regulating this cycling are crucial for rab function. However, an essential role for rab nucleotide exchange factors has not yet been demonstrated. In this report we show the requirement of nucleotide exchange factor activity for Ypt1 GTPase mediated protein transport. The Ypt1 protein, a member of the rab family, plays a role in targeting vesicles to the acceptor compartment and is essential for the first two steps of the yeast secretory pathway. We use two *YPT1* dominant mutations that contain alterations in a highly conserved GTP-binding domain, *N121I* and *D124N*. *YPT1-D124N* is a novel mutation that encodes a protein with nucleotide specificity modified from guanine to xanthine. This provides a tool for the study of an individual rab GTPase in crude extracts: a xanthosine triphosphate (XTP)-dependent conditional dominant mutation. Both mutations confer growth inhibition and a block in protein secretion when expressed in vivo. The purified mutant proteins do not bind either GDP or GTP. Moreover, they completely inhibit the ability of the exchange factor to stimulate nucleotide exchange for wild type Ypt1 protein, and are potent inhibitors of ER to Golgi transport in vitro at the vesicle targeting step. The inhibitory effects of the Ypt1-D124N mutant protein on both nucleotide exchange activity and protein transport in vitro can be relieved by XTP, indicating that it is the nucleotide-free form of the mutant protein that is inhibitory. These results suggest that the dominant mutant proteins inhibit protein transport by sequestering the exchange factor from the wild type Ypt1 protein, and that this factor has an essential role in vesicular transport.

THE passage of proteins through the secretory pathway involves their orderly progression through a series of membranous compartments (Palade, 1975). Transport between successive secretory compartments appears to be mediated by vesicles (Jamieson and Palade, 1967; Pfeffer and Rothman, 1987; Rothman, 1994). However, the mechanisms that regulate the directionality and specificity of these vesicular transport steps are not known. The rab/Ypt1/Sec4 family of small GTPases has been shown to play a role in vesicular trafficking (Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993). It has been suggested that these proteins act at the different steps of the secretory pathway to ensure the fidelity of vesicular targeting (Bourne, 1988; Segev et al., 1988; Lian et al., 1994; Søgarrd et al., 1994). The ability of rab proteins to cycle between GTP- and GDP-bound forms is believed to be crucial for their function (Bourne, et al., 1990). The shift from the GTP- to the GDP-bound form is accomplished by the endogenous GTPase activity of these proteins, while the change from the GDP- to the GTP-bound form is achieved by nucleotide exchange. Most GTP-binding proteins have slow intrinsic rates of guanine nucleotide exchange, and thus require accessory factors to stimulate the exchange reaction.

The importance of nucleotide exchange for function of rab-GTPases has been implied in several studies. First, rab proteins that have a mutation analogous to ras-S17N, which locks the GTPase in the GDP-bound form, have been shown to inhibit protein transport in vivo and in vitro (Tisdale et al., 1992; Li and Stahl, 1993; Nuoffer et al., 1994; Riederer et al., 1994; Stenmark et al., 1994). Second, the bulk of the cytosolic pool of several rab proteins is apparently in the GDP-bound state, since it is found in a complex with GDP-dissociation inhibitory factor (GDI)[1]

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; EF, elongation factor; 5-FOA, 5-fluoro-orotic acid; GAP, GTPase-activating protein; GDS, GDP dissociation stimulators; GDI, GDP-dissociation inhibitory factor; S-raffinose, synthetic-raffinose; Ypt1p, Ypt1 protein; XTP, xanthosine triphosphate.

(Regazzi et al., 1992; Soldati et al., 1993; Ullrich et al., 1993), which has been shown to associate only with the GDP-bound form of rab proteins (Araki et al., 1990). Third, a temporal correlation has been found between the association of rab5 and rab9 with membranous compartments and binding of GTP to these proteins (Soldati et al., 1994; Ullrich et al., 1994). Thus, it has been suggested that guanine nucleotide exchange is crucial for rab protein function, and may play a role in recruitment of the protein to its target membrane.

Factors that stimulate nucleotide exchange have been identified for members of the ras and rho families (Boguski and McCormick, 1993) and also for elongation factor (EF) Tu (Kaziro, 1978). Less is known about exchange factors for rab-GTPases. To date, three GDP dissociation stimulators (GDS) for rab proteins have been described. The yeast *DSS4* and the mammalian *mss4* genes were identified based on their ability to suppress a recessive *sec4* mutation in yeast (Moya et al., 1993; Burton et al., 1993, respectively). The third factor, Rab3A-GRF (guanine-nucleotide release factor), is a mammalian protein that was identified as a biochemical activity which stimulates GDP release from the Rab3A protein (Burstein and Macara, 1992). Even though their specificity appears to overlap, Rab3A-GRF and Mss4 were suggested to be two distinct factors based on the fact that unlike Mss4, Rab3A-GRF is more effective on the lipid-modified form of Rab3A than on its unmodified form (Miyazaki et al., 1994). Deletion of *DSS4* is not lethal to yeast cells and does not seem to affect cell growth or secretion, despite the fact that *SEC4* function is essential for viability and secretion (Moya et al., 1993). This observation raises the questions of whether Dss4p is the only exchange factor acting on Sec4p and, more importantly, whether guanine nucleotide exchange factors are essential for rab function. We have identified an exchange factor for Ypt1 protein (Ypt1p) in yeast. This factor is distinct from Dss4p, and stimulates both GDP release and GTP uptake by Ypt1p (Jones, S., R. J. Litt, and N. Segev, manuscript in preparation). In the present paper, we explore the role of this nucleotide exchange factor by taking advantage of Ypt1 dominant mutant proteins that inhibit its activity.

We used two dominant interfering mutations of *YPT1* that change residues in a guanine-binding domain that is highly conserved in all GTPases. The first mutation is *YPT1-N121I* (Schmitt et al., 1986). A corresponding mutation has been studied in several rab proteins in yeast and mammalian cells. Mutant rab proteins carrying this mutation have been shown to interfere with protein transport in vivo and in vitro and to have a reduced affinity for guanine nucleotides (Wagner et al., 1987; Walworth et al., 1989; Gorvel et al., 1991; Bucci et al., 1992; Tisdale et al., 1992; Brondyk et al., 1993). The mechanism by which this mutation exerts its dominant effect in rab proteins has yet to be established. The second mutation, *YPT1-D124N*, is analogous to the EF-Tu mutation D138N, which has been shown to confer a defect in nucleotide binding (Hwang and Miller, 1987). Two other EF-Tu mutants with alterations in the same consensus domain, K136E/Q, are also defective in nucleotide binding. These two mutant proteins exert their dominant negative effects by sequestering EF-Ts, the exchange factor for EF-Tu (Hwang et al., 1989). A similar mechanism of action has been demonstrated for *ras* dominant interfering mutations in this and another GTP-binding consensus domain (Powers et al., 1989, 1991; Hwang et al., 1993; Lai et al., 1993; Haney and Broach, 1994).

In this study, we used dominant interfering mutants of the Ypt1-GTPase to assess the role of its exchange factor in protein transport. Ypt1 protein, a member of the rab family, is essential for the first two steps of the yeast secretory pathway, ER to Golgi, and *cis*- to medial-Golgi apparatus, mediating the targeting and/or fusion of transport vesicles (Segev et al., 1988; Bacon et al., 1989; Rexach and Schekman, 1991; Segev, 1991; Jedd, G., C. J. Richardson, R. J. Litt, and N. Segev, manuscript in preparation). The two dominant mutant Ypt1 proteins, Ypt1-N121I and Ypt1-D124N, fail to bind guanine nucleotides, and they inhibit protein transport in vivo and in vitro. They do so by blocking the activity of the exchange factor for Ypt1p, thus demonstrating the importance of this factor in secretion. *YPT1-D124N* is a novel mutation, which by analogy to a similar mutation in EF-Tu is predicted to change the specificity of nucleotide binding from guanosine phosphates to xanthosine phosphates (Hwang and Miller, 1987). The *D124N* mutation has the same effect on nucleotide specificity in the Ypt1p. As a consequence, the inhibitory effects of the Ypt1 mutant protein in the exchange and in vitro transport assays can be alleviated by providing XTP. It is likely that the *D124N* mutation will have a similar effect on other rab proteins, thus providing a new tool for the study of specific rab proteins in crude extracts. In addition, since the mutant proteins seem to exert their dominance by sequestering the exchange factor, they are potential tools for the isolation of these factors.

Materials and Methods

Strains, Plasmids, and Materials

The following yeast strains were used in this study: GPY60 (*MATα ura3-52 trp1 leu2 his4 pep4::URA3*), NSY181 (*MATa/α his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 ura3-52/ura3-52*), NSY125 (DBY1034; *MATa his4-539 lys2-801 ura3-52*). Yeast and bacterial transformations were performed by electroporation (Dower et al., 1988; Becker and Guarente, 1991).

Plasmids were constructed for the inducible expression of wild type and mutant *YPT1* alleles in yeast. They contain the EcoRV to BamHI fragment containing the entire *YPT1* open reading frame inserted into pRS316 (*CEN-URA3*) (Sikorski and Hieter, 1989) and the *GAL10* promoter (Johnston and Davis, 1984). Mutant *YPT1* alleles were constructed by site directed mutagenesis as described below. Yeast expression plasmids were designated pRS326, wild type *YPT1*; pRS327, *YPT1-N121I* and pRS317, *YPT1-D124N*. Yeast strains carrying these plasmids express Ypt1p when grown on medium containing galactose.

Plasmids for the expression of GST fusion proteins in *Escherichia coli* were constructed in pGEX-KT (Hakes and Dixon, 1992) by inserting BamHI fragments containing the entire *YPT1* open reading frame from pRS351 (wild type *YPT1*), pRS360 (*YPT1-N121I*), or pRS318 (*YPT1-D124N*) (see below) into the BamHI site of the vector. Bacterial plasmids for the expression of the GST-Ypt1 fusion proteins were designated pRS361, wild type Ypt1p; pRS362, Ypt1p-D124N, and pRS363 Ypt1p-N121I. Thrombin cleavage of the fusion proteins yields Ypt1 protein with a two amino acid extension (Gly-Ser) appended to the amino terminus.

All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted.

Culture Conditions

Yeast strains were grown in rich medium (YEP, 1% yeast extract, 2% bac-

topeptone) or synthetic medium (0.67% yeast nitrogen base without amino acids) supplemented with the appropriate auxotrophic requirements (Rose et al., 1988). Unless otherwise noted, carbon sources were added to 2% (wt/vol).

Site-directed Mutagenesis

YPT1 mutations were made in the *E. coli* phagemid vector pIBI31 (International Biotechnologies, Inc., New Haven, CT) containing the EcoRI to BamHI fragment of *YPT1*. Mutants were constructed by the method of Kunkel (1987) and tested in strain NSY125. Constructs were designated pRS300, wild-type *YPT1*; pRS301, *YPT1-N121I* and pRS318 *YPT1-D124N*. Mutagenic oligonucleotides were: *YPT1-N121I*: AAG CTA TTG GTA GGT ATC AAG TGT GAT TTA AAG; or *YPT1-D124N*: GTA GGT AAC AAG TGT AAT TTA AAG GAC AAG CGT. Mutant alleles were confirmed by sequence analysis using Sequenase (United States Biochemical, Cleveland, OH).

Random Mutagenesis

Plasmid pRS326 (wild type *YPT1*) was mutagenized in vitro with hydroxylamine as described (Rose et al., 1988). The frequency of mutagenesis was 2% as determined by the loss of *URA3* function in bacteria. Mutagenized plasmids were introduced into the *YPT1* wild type strain NSY181. Ura^+ yeast transformants were selected and screened for their inability to grow in the presence of galactose. Strains that were unable to grow on galactose were considered potential dominant mutants. Those that regained the ability to grow on galactose following 5-fluoro-orotic acid (5-FOA) selection (Boeke et al., 1987) were retained. Plasmids containing candidate mutations were isolated as described (Hoffman and Winston, 1987). The identity of the *YPT1* mutation was determined by sequence analysis. A screen of 12,000 yeast transformants yielded two dominant lethal alleles of *YPT1*. Both of these alleles were novel. The screen was not saturating since it failed to yield a previously identified dominant interfering mutation (Schmitt et al., 1986).

In Vivo Protein Labeling and CPY Immunoprecipitation

Derivatives of strain NSY125 transformed with pRS326-based yeast expression vectors were grown to 0.2 A_{600} U/ml, in synthetic-raffinose (S-raffinose) -URA dropout medium. D-galactose was added to a final concentration of 2% (wt/vol) and cells were incubated at 30°C for 3 h before labeling. Cells were pelleted and resuspended to 5 A_{600} U/ml in S-raffinose-URA medium with 150 μCi/ml Tran^{35}S-label (~1,000 Ci/mmol; ICN Biochemicals, Inc., Irvine, CA). Metabolic labeling was carried out at 30°C for 10 min. Cells were pelleted and chase was initiated by resuspension in S-raffinose-URA medium with 0.003% L-cysteine and 0.004% L-methionine (wt/vol). Samples of 0.2 ml were taken at intervals and placed on ice; sodium azide was added to 10 mM. Cells were pelleted and resuspended in 0.1 ml of lysis buffer (50 mM Tris, pH 7.5, 6 M urea, 1% SDS, 1 mM EDTA). An equal volume of glass beads was added and samples were vortexed for 1 min and boiled for 5 min. 0.9 ml dilution buffer (50 mM Tris, pH 7.5, 1.25% Triton X-100, 190 mM NaCl, 6 mM EDTA) was added and samples were centrifuged at 16,000 *g* for 10 min at 4°C. Lysates were pre-cleared with 20 μl of a 50% slurry of protein A–Sepharose pre-bound to rabbit anti–mouse IgG (Zymed Labs, San Francisco, CA). CPY was immunoprecipitated with mouse anti–CPY monoclonal antibody and immunocomplexes were washed as described (Graham and Emr, 1991), electrophoresed on 12% SDS-PAGE (Laemmli, 1970), processed for fluorography, and visualized by autoradiography.

Purification of Mutant Ypt1 Proteins

GST fusion proteins were purified as described (Smith and Johnson, 1988) except that induction was carried out at 25°C, 10 mM $MgCl_2$ was included in all steps and binding to glutathione agarose was carried out at 4°C for 1 h. Following thrombin cleavage, Ypt1p-containing fractions were pooled and dialyzed against buffer 88 (250 mM sorbitol, 20 mM Hepes, pH 6.8, 150 mM KOAc, 5 mM $Mg[OAc]_2$) (Baker et al., 1988).

Guanine Nucleotide Binding Assays

Ypt1 protein (50 μg/ml) in buffer 88 was incubated at 30°C with 3.2 mM ^{3}H-5′,8′-GDP (New England Nuclear, Boston, MA) or ^{32}P-α-GTP (Amersham Corp., Arlington Heights, IL) diluted to a specific activity of approximately 15 Ci/mmol and EDTA to bring the free concentration of Mg^+ to ~0.1 μM (Sunyer et al., 1984). Samples containing 0.1 μg Ypt1p were removed at intervals and were applied to wet nitrocellulose filters (BA85; Schliecher & Schuell, Keene, NH) which were then washed twice with 3 ml of ice cold wash buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT). Background was assessed by a parallel sample that contained BSA in place of Ypt1p. Radioactivity bound to the filters was quantified by liquid scintillation spectrometry using Ready Protein+ (Beckman Instruments, Palo Alto, CA) or Filtron-X (National Diagnostics, Atlanta, GA) scintillation fluid. Mutant proteins never bound nucleotides above background, and the given estimates express the lowest detection limit of the assay.

GDP Release Assays

20 pmol Ypt1p were preloaded by incubating with 40 pmol 5′,8′-^{3}H-GDP (S.A. 31.7 Ci/mmol; New England Nuclear) in preload buffer (20 mM Hepes, pH 7.2, 20 mM KOAc, 1 mM DTT, 5 mM EDTA) for 10 min at 30°C. At the end of the incubation, samples were moved to ice and $MgCl_2$ was added to 5 mM. ^{3}H-GDP remained stably bound to the Ypt1p treated in this way for at least 1 h. The P100 cell fraction (Baker et al., 1990) which contains the stimulating exchange activity for Ypt1p or BSA (unstimulated control) were diluted into reaction buffer (20 mM Hepes, pH 7.2, 5 mM $Mg[OAc]_2$, 0.75 mM GTP, 0.75 mM GDP, 1 mM DTT) and exchange reactions were initiated by the addition of 10 pmol Ypt1p-^{3}H-GDP. The reaction volume was 50 μl. Incubations were carried out at 30°C for varying periods of time, as noted. To monitor the release of ^{3}H-GDP from Ypt1p, 5 μl samples were filtered, washed and counted as above. In all experiments, initial values were approximately 8–10 × 10^3 dpm per 5-μl sample (Jones, S., R. J. Litt, and N. Segev, manuscript in preparation).

GTP Uptake Assays

Bacterially expressed Ypt1p was preloaded as described for the GDP release assay, but with non-radioactive GDP. As a source of exchange activity we used a cellular fraction from which the endogenous Ypt1p was extracted. The P100 fraction was treated with 1% Triton X-100 for 1 h on ice. Following centrifugation at 100,000 *g* for 1 h, the pellet, which contained ~90% of the exchange activity, was resuspended in buffer 88 and further extracted with 0.5 M NaCl for 1 h on ice. A final centrifugation at 100,000 *g* for 1 h yielded a supernatant (termed TNS100) which contained ~75% of the total exchange activity in the original P100 fraction (Jones, S., R. J. Litt, and N. Segev, manuscript in preparation). The preloaded Ypt1p and TNS100 fraction or ovalbumin (unstimulated control) were added to reaction buffer lacking GTP or GDP. ATP was added to 1 mM to prevent hydrolysis of ^{32}P-GTP. α-^{32}P-GTP (Amersham Corp.; 3,000 Ci/mmol) was diluted to a specific activity of 75 Ci/mmol. Exchange reactions were initiated by the addition of 100 pmol GTP to a 50 μl reaction mixture containing 10 pmol Ypt1p and 20 μg TNS100. Samples of 5 μl were removed at intervals and the amount of α-^{32}P-GTP bound to Ypt1p was determined by quantitative immunoprecipitation with anti-Ypt1p antibodies. Immunoprecipitation was necessary to distinguish between Ypt1p and other GTP-binding proteins present in the TNS100 extract (Jones, S., R. J. Litt, and N. Segev, manuscript in preparation).

ER to Golgi Transport Assay

In vitro ER to Golgi transport assays were carried out as described (Baker et al., 1988, 1990). Membranes (Baker et al., 1990) and cytosol (Wuestehube and Schekman, 1992) were prepared as described.

Results

Isolation and In Vivo Characterization of a New Dominant Lethal Allele of YPT1

We wished to identify new dominant *YPT1* mutations which, by analogy to EF-Tu and *RAS2* (see above), might prove to be valuable for analysis of the exchange factor that acts on Ypt1p. Since we expected that such mutations would be lethal, the *Saccharomyces cerevisiae YPT1* gene

was placed under control of the inducible *GAL10* promoter. We identified several new dominant lethal alleles of *YPT1* by random or site-directed mutageneses. One of the mutations identified by both methods resulted in the substitution of an asparagine residue in place of an aspartate at position 124 of Ypt1p. This allele, designated *YPT1-D124N*, is a new allele of *YPT1* that is similar to existing mutations in EF-Tu (Hwang and Miller, 1987) and the human (Feig et al., 1986; McCormick et al., 1987) and *Caenorhabditis elegans* (Han and Sternberg, 1991) *ras* genes. A dominant mutation in a neighboring residue, *YPT1-N121I*, was originally isolated by Schmitt et al. (1986) and is similar to existing mutations in *ras* (Clanton et al., 1986; Der et al., 1986; Walter, et al., 1986) and in other rab proteins (Walworth et al., 1989; Gorvel et al., 1991; Tisdale et al., 1992; Brondyk et al., 1993). Expression of either *YPT1-D124N* or *YPT1-N121I* in yeast cells caused growth arrest (Fig. 1 *a*; and Schmitt et al., 1986). Both mutant phenotypes exhibited some temperature dependence, with the lethal effects being more pronounced at low temperatures (data not shown). Unlike the behavior of the *SEC4-N133I* mutation, which is analogous to *YPT1-N121I* (Walworth et al., 1989), attempts to transform cells with *CEN* plasmids carrying either *YPT1* mutant allele under the control of its own promoter were unsuccessful. These results suggest that even low levels of expression are sufficient to confer the dominant lethal effect. Replacement of two terminal cysteine residues, which are necessary for prenylation, with serine residues did not alleviate the lethal phenotype of *YPT1-N121I* when the doubly mutant allele was expressed under control of the *GAL10* promoter (data not shown). Thus, the dominant effect of *YPT1-N121I* in vivo is apparently not dependent upon prenylation. This feature of the dominant mutant protein encouraged us to use a bacterially produced unprenylated protein for further biochemical analysis.

Loss of Ypt1p activity results in a defect in the first two steps of the yeast secretory pathway (Segev et al., 1988; Bacon et al., 1989; Jedd, G., R. J. Litt, C. J. Richardson, and N. Segev, manuscript in preparation). Yeast cells expressing the Ypt1-N121I or -D124N dominant mutant proteins exhibit similar defects in protein transport in vivo. The effect of expressing the mutant proteins on vesicular transport was assayed by following the processing of the vacuolar protein carboxypeptidase Y (CPY). As CPY traverses the secretory pathway it progresses from a core glycosylated, ER form (p1), to an outer-chain glycosylated, Golgi form (p2), and finally to a proteolytically processed, mature vacuolar form (m). Pulse–chase experiments, followed by immunoprecipitation of CPY, were performed using strains carrying galactose-inducible wild type *YPT1*, *YPT1-N121I*, or *YPT1-D124N* alleles (Fig. 1 *b*). Cells expressing the wild type Ypt1 protein, or cells carrying the *YPT1-N121I* or *-D124N* allele but grown under non-inducing conditions (glucose), process ~90% of the CPY to the mature form within 10 min of chase (Fig. 1 *b*, and data not shown). Expression of either dominant mutant allele significantly slows transport of CPY through the secretory pathway, with the p1 form and a broad band above it persisting even after an hour of chase (Fig. 1 *b*). A more detailed characterization of the effect of *YPT1-N121I* on CPY transport showed that the immature CPY forms that accumulate in these cells are the ER and *cis*-Golgi forms (Jedd, G., C. J. Richardson, R. J. Litt, and N. Segev, manuscript in preparation). In the time course of the secretion experiment (4 h) there was no effect on cell

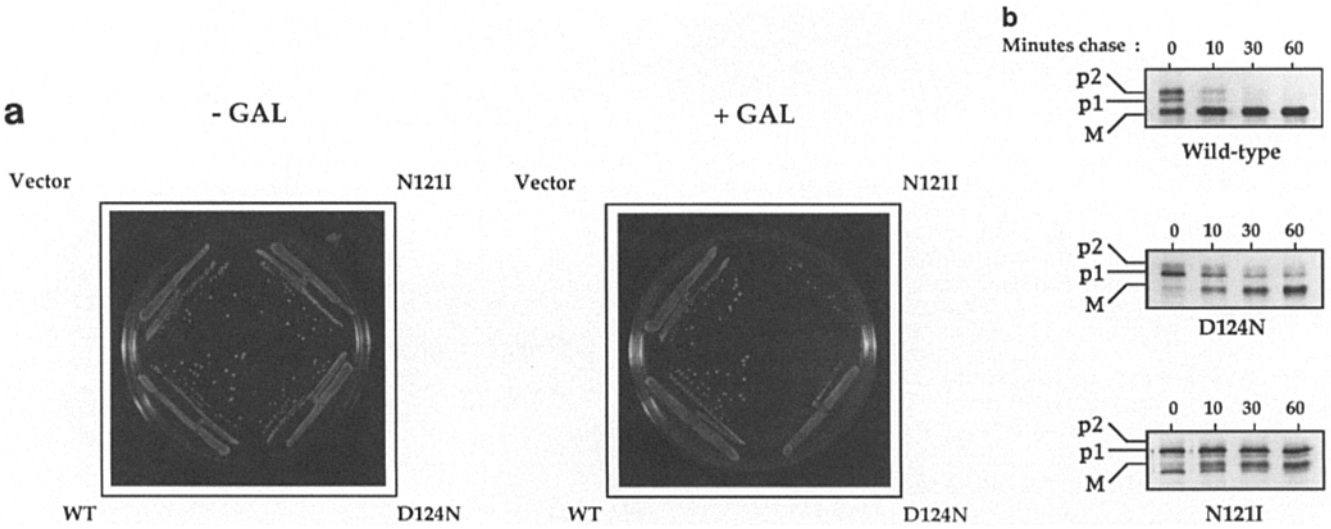

Figure 1. The dominant *YPT1* mutations *N121I* and *D124N* cause growth inhibition and a protein secretion defect in vivo. (*a*) Expression of these dominant negative mutants inhibits cell growth. Yeast cells (NSY125) transformed with empty vector or plasmids carrying galactose-inducible wild type or mutant *YPT1* alleles were streaked on synthetic raffinose medium (S-raffinose-URA) without (*left*) or with (*right*) 2% galactose. (*b*) Expression of dominant negative mutants perturbs the secretory pathway. Yeast cells (NSY125) transformed with plasmids carrying wild type or mutant *YPT1* alleles were grown to early log phase in S-raffinose-URA. Wild type or mutant Ypt1 protein were induced by the addition of 2% galactose for 3 h. The cells were pulse labeled with ^{35}S-translabel for 10 min at 30°C, and then chased with excess cold methionine and cysteine. Samples were removed at indicated chase time points and CPY was immunoprecipitated from whole cell lysates. Samples were analyzed by SDS-PAGE and fluorography. The secretory phenotype is apparent already after the pulse, chase time 0, because the mutant alleles were induced for 3 h prior to labeling.

viability as judged by vital staining of yeast cells using propidium iodide (Corliss and White, 1981) (data not shown). The growth and secretion phenotypes conferred by the *YPT1-N121I* allele were more severe than those caused by *YPT1-D124N*. Western blot analysis showed that levels of Ypt1 protein expression were similar in strains expressing either allele (data not shown). Since the two proteins are equally potent inhibitors in vitro (see below), we suggest that the observed difference in vivo was due to the presence of xanthine nucleotides in the cell (Gardner and Woods, 1979), which can relieve the defect caused by Ypt1p-D124N but not by Ypt1p-N121I (see below).

Dominant Mutant YPT1 Proteins Are Defective in Guanine Nucleotide Binding

The *YPT1-D124N* and *YPT1-N121I* mutations lie in a domain of the protein that, by analogy to ras, is predicted to interact with the guanine nucleotide ring (Bourne et al., 1991). This domain and the aspartate and asparagine residues are highly conserved among all small GTP-binding proteins. We therefore examined the capacity of the wild type and mutant Ypt1 proteins to bind GDP and GTP. Nucleotide binding experiments using Ypt1 proteins and ^{3}H-GDP were carried out, varying the molar ratio of GDP to protein from 1:1 to 50:1. While nucleotide binding by wild type Ypt1p was clearly detectable (at least 80-fold above the background), no significant binding by mutant proteins was detected at even the highest GDP to protein ratio (data not shown). Since magnesium is necessary for strong guanine nucleotide binding, the rate of intrinsic nucleotide exchange can be accelerated dramatically by titration of magnesium with EDTA (Hall and Self, 1986). We therefore determined nucleotide binding in the presence of EDTA. Wild type Ypt1p bound GDP and GTP equally well under these conditions. In contrast, the Ypt1-N121I and -D124N mutant proteins still failed to show significant binding to either GDP or GTP (Table I). Failure to bind nucleotides does not seem to affect the stability of these proteins for two reasons. Most importantly, these proteins inhibit the exchange factor for Ypt1p and protein transport in vitro; both effects can be relieved by XTP in the case of Ypt1-D124N (see below). In addition, the mutant proteins migrate appropriately according to their molecular mass, like the wild type protein, using a Sepharose S300 gel filtration column followed by immunoblot analysis (data not shown).

Dominant Mutant Ypt1 Proteins Completely Inhibit Stimulated Guanine Nucleotide Exchange for Wild Type Ypt1p

Genetic and biochemical evidence suggests that certain dominant interfering EF-Tu and ras mutant proteins (e.g., EF-Tu-Q136E/K, Ras2p-G22A, H-ras-N116N), which are defective in guanine nucleotide binding, sequester their exchange factors, EF-Ts and Cdc25/Sdc25, respectively (Hwang et al., 1989; Powers et al., 1989; Lai et al., 1993; Hwang et al., 1993). Ras proteins carrying these mutations have been shown to interfere with the ability of Cdc25 or Sdc25 to stimulate the exchange of guanine nucleotides bound to wild type ras protein (Hwang et al., 1993; Haney and Broach, 1994). We have recently characterized a guanine nucleotide exchange activity for Ypt1p. This exchange factor is different from the other known exchange factor for rab proteins in yeast, Dss4p, since it is undiminished by deletion of the *DSS4* gene (Jones, S., R. Litt, and N. Segev, manuscript in preparation). We wished to examine whether the dominant interfering Ypt1 mutant proteins, Ypt1-N121I and Ypt1-D124N, which are defective in guanine nucleotide binding, can block this exchange activity.

Table I. Ypt1 Dominant Mutant Proteins Are Defective in Guanine Nucleotide Binding

	Nucleotide Binding (pmol/pmol Ypt1p)	
Ypt1 Protein	GDP	GTP
wild type	0.215	0.223
D124N	<0.0001	<0.0002
N121I	<0.0001	<0.0002

Wild type and mutant Ypt1 proteins (2 μM) were incubated with ^{3}H-5′,8′-GDP or ^{32}P-α-GTP (3.2 μM) for 15 min at 30°C in the presence of sufficient EDTA to bring the free Mg^+ concentration to 0.1 μM (Sunyer et al., 1984). Magnesium (5 mM) was then added to stabilize the nucleotide–protein complex. Samples were removed, filtered onto nitrocellulose, and quantitated by scintillation spectrophotometry. The data shown represent the averages of three independent determinations and agree to within 11% of the mean.

Exchange of guanine nucleotide bound to wild type Ypt1p was assayed by GDP release or GTP uptake. The P100 fraction (100,000 *g* pellet) of a crude yeast lysate was found to be enriched in a nucleotide exchange stimulating activity for Ypt1p. The P100 fraction or an extract of it, TNS100, were used in the following experiments as the source of this activity. Exchange assays were performed to follow stimulated release of ^{3}H-GDP from preloaded wild type Ypt1p, in the presence of varying amounts of wild type or mutant Ypt1 proteins. Addition of bacterially-expressed purified Ypt1-N121I and Ypt1-D124N proteins inhibited the activity of the exchange factor in stimulating the release of ^{3}H-GDP from Ypt1p, in a concentration dependent manner (Fig. 2 *a*). Both mutant proteins also inhibited stimulated uptake of ^{32}P-GTP by wild type Ypt1p (Fig. 2 *b*). The inhibitory characteristics of the two mutant proteins were identical; 50% of maximal inhibition was achieved at 0.07 μM inhibitory protein and complete inhibition at 0.3 μM. In contrast to the mutant proteins, wild type Ypt1p did not inhibit stimulated exchange, even when added at a concentration of up to 3 μM (Fig. 2 *a*). The inability of wild type Ypt1p to inhibit the exchange reaction even at high concentrations suggests that the interaction between the wild type Ypt1p and the exchange factor is transient. In contrast, the ability of the mutant proteins to inhibit the exchange reaction at a very low concentration suggests that the interaction between the mutant proteins and the exchange factor is more stable. We suggest that the difference between the mutant and the wild type Ypt1 proteins is due to the fact that while the mutant proteins are nucleotide-free, the wild type Ypt1p is either nucleotide bound, or capable of binding nucleotides, which are present in excess in the assay mixture. The effect of the mutant proteins was limited to stimulated guanine nucleotide exchange; the intrinsic (uncatalized) exchange rate, which was about eightfold lower than the stimulated exchange rate, was not affected (data not shown). This observation strongly suggests that the mutant protein does not complex with the wild type Ypt1p in such

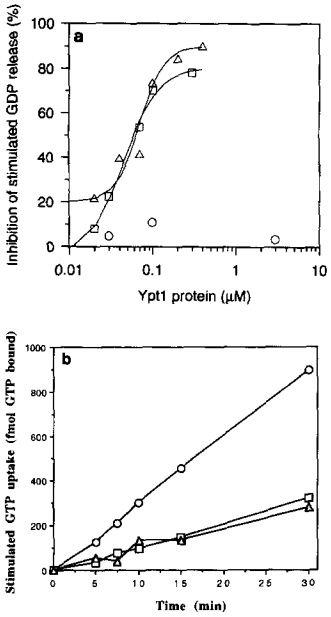

Figure 2. Ypt1-N121I and -D124N mutant proteins completely inhibit stimulated guanine nucleotide exchange on wild type Ypt1p. (*a*) Inhibition of stimulated GDP release. Guanine nucleotide exchange was monitored by measuring the release of ^{3}H-GDP from Ypt1p. Reactions were supplemented with increasing amounts of wild type Ypt1 (*circles*), Ypt1-N121I (*triangles*), or Ypt1-D124N (*squares*) proteins expressed in bacteria. Assays were performed by preincubating competitor Ypt1 proteins with the P100 cellular fraction (1 μg/ml) for 10–15 min on ice. Wild-type Ypt1 protein (0.1 μM) preloaded with ^{3}H-GDP was then added as a substrate and the reaction was incubated at 30°C for 30 min. Samples were removed, filtered onto nitrocellulose, and quantitated by scintillation spectrophotometry. Values presented are the average of 2–4 experiments. Inhibition of stimulated exchange is expressed as inhibition of GDP release caused by the addition of Ypt1p to the stimulated reaction elicited by the P100 fraction (42% GDP release). Intrinsic exchange (6% GDP release), measured in a control reaction, in which BSA was added instead of P100, was subtracted. (*b*) Inhibition of stimulated GTP uptake. Guanine nucleotide uptake was monitored by measuring stimulated binding of α-^{32}P-GTP to Ypt1p. Reactions were carried out in the absence (*circles*) or presence of 0.2 μM Ypt1-N121I (*triangles*), or Ypt1-D124N (*squares*) proteins purified from bacteria. Assays were performed as in Fig. 2 *a*, except that the source of exchange factor was the TNS100 extract of the P100 fraction and the amount of GTP bound to Ypt1p was determined by immunoprecipitation rather than filtration. Values presented are the average of two experiments and are representative of four experiments using two extracts. Intrinsic nucleotide exchange (assayed in the presence of ovalbumin) was measured at each time point and has been subtracted out to generate the values for stimulated exchange. Stimulated GTP uptake was inhibited 65% under these conditions. In ^{3}H-GDP release assays carried out with TNS100, under identical conditions, exchange was inhibited 60%.

a way as to prevent release or binding of guanine nucleotides. Instead, it supports the model that inhibition of exchange by the mutant proteins is due to interference with the interaction between wild type Ypt1p and the exchange factor.

Dominant Mutant Ypt1 Proteins Inhibit ER to Golgi Transport In Vitro at the Vesicle Consumption Step

Ypt1p has been shown to be required for transport of proteins in a cell-free system that reconstitutes ER to Golgi transport (Bacon et al., 1989; Baker et al., 1990). If stimulated nucleotide exchange is essential for Ypt1p function in protein transport, the bacterially expressed dominant mutant proteins, which inhibit this process, should inhibit

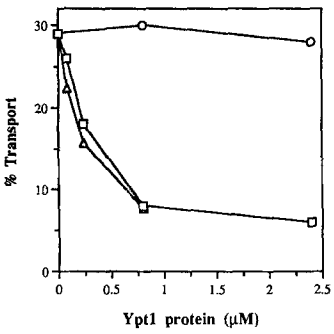

Figure 3. Ypt1-N121I and Ypt1-D124N dominant mutant proteins inhibit ER to Golgi transport in vitro. ER to Golgi transport of ^{35}S-labeled pro-α-factor was assayed in a cell-free reaction (Baker, et al., 1988). The indicated amounts of purified recombinant Ypt1 proteins (wild type Ypt1, *circles*; Ypt1-N121I, *triangles*; Ypt1-D124N, *squares*) were added. Percent transport was calculated as percent of ER modified (core-glycosylated, Concanavalin A [Con A] precipitable cpm) α-factor that acquired Golgi-specific modification (anti-α-1,6 mannose precipitable cpm). Concentration of mutant protein required for 50% inhibition of transport varied among different preparations of fractions used in the reaction. Data presented are the average of three determinations using one set of fractions.

ER to Golgi transport in vitro. Wild type and mutant Ypt1 proteins purified from *E. coli* were added to a reaction in which transport of a labeled protein is assayed by following the addition of compartment-specific modifications (Baker et al., 1988, 1990). Both mutant proteins were potent inhibitors of ER to Golgi transport (Fig. 3). 50% inhibition was achieved with 0.3 μM Ypt1p-N121I or Ypt1p-D124N; maximal inhibition of ~75–85% was achieved by adding 0.8 μM mutant proteins. In contrast, addition of comparable amounts of wild type Ypt1p had no effect on transport. Thus, the mutant proteins are capable of acting as dominant inhibitors of protein transport in vitro as well as in vivo. Inhibition of the in vitro transport reaction was achieved at concentrations of mutant proteins that are in the same range as those required for inhibition of the exchange activity. Since the proteins were purified from a bacterial expression system, prenylation appears to be unnecessary for this inhibitory effect, as is also the case in vivo (see above).

Ypt1p is required for targeting and/or fusion of transport vesicles on their way to the Golgi complex from the ER. This was shown by the fact that inhibiting Ypt1p function, using either antibodies or fractions made from cells carrying a recessive *ypt1* mutation, blocks the transport reaction at a step subsequent to vesicle budding from the ER (Rexach and Schekman, 1991; Segev, 1991). If the dominant mutant protein causes the block in transport by interfering with wild type Ypt1p function, it is expected to block at the same step as the antibody or the recessive *ypt1* mutation, namely after vesicle budding. To determine whether the dominant mutant proteins inhibited transport at this step, the Ypt1p-D124N-blocked in vitro transport reaction was assayed for accumulation of transport vesicles. This assay involved determining whether an ER-modified marker protein, lacking Golgi modifications, accumulated in the supernatant after a medium-speed centrifugation (Rexach and Schekman, 1991; Segev, 1991). Even though protein transport to the Golgi complex was 85% blocked by the addition of Ypt1p-D124N, exit from the ER was as high as in control reactions (Fig. 4 *a*). The labeled marker protein that accumulated in the superna-

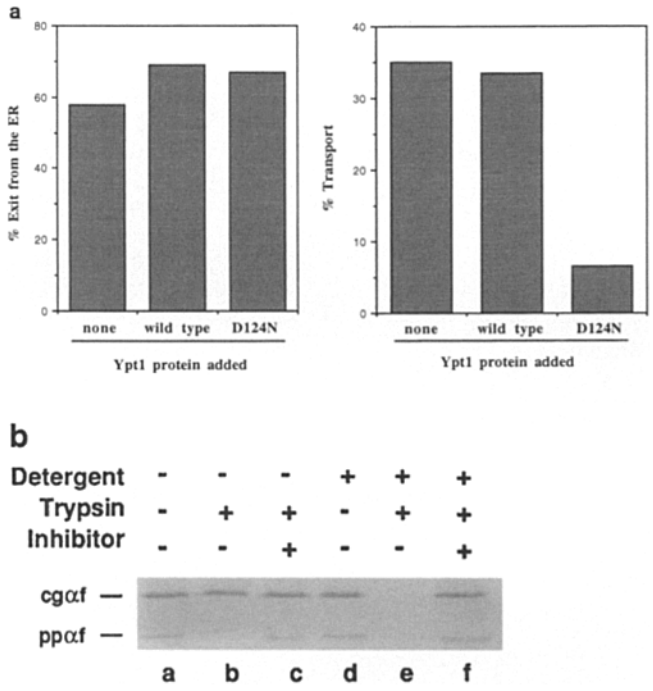

Figure 4. The Ypt1-D124N mutant protein blocks the in vitro ER to Golgi transport reaction at a step after vesicle budding. (*a*) Exit from the ER is not blocked by the Ypt1-D124N mutant protein. ER to Golgi in vitro transport of ^{35}S-labeled α-factor was assayed as described for Fig. 3. Either no recombinant protein, or 2.4 μM wild type Ypt1 or Ypt1-D124N was added to the in vitro reaction. Samples were removed after incubation at 20°C for 60 min and fractionated by differential centrifugation. Samples were assayed for ER modified or Golgi modified α-factor in the total reaction mixture and in the medium speed supernatants (MSS, 12,000 *g* for 10 min) (Segev, 1991). The percent exit from the ER is calculated as the sum of the ER modified α-factor in the MSS plus Golgi modified α-factor in the total reaction, versus the total α-factor tanslocated into the ER (Con A precipitable cpm in the unfractionated reaction) (*left*). Percent transport (*right*) is calculated as in Fig. 3. Values presented are representative of three experiments. (*b*) ER modified α-factor accumulated in the Ypt1-D124N-blocked in vitro transport reaction is enclosed within membranes. The ER to Golgi in vitro transport reaction was carried out in the presence of Ypt1-D124N protein as in *a*. Medium speed supernatant containing intermediate vesicles was treated with trypsin in the absence (lanes *a–c*) or presence (lanes *d–f*) of detergent as described (Segev, 1991). After protease treatment, samples were subjected to immunoprecipitation with antibody to α-factor, followed by SDS PAGE and fluorography (*cgα f*, core glycosylated α-factor; *ppα f*, pre-pro-α factor). Core glycosylated α-factor was accessible to protease only when detergent was included; protease digestion was abolished by addition of trypsin inhibitor (lanes *c* and *f*). Pre-pro-α factor is the untranslocated form of the marker protein and is accessible to protease even in the absence of detergent.

tant was protease resistant in the absence but not in the presence of detergent, demonstrating that it was enclosed within membranes (Fig. 4 *b*). Thus, Ypt1-D124N protein blocks transport at a point subsequent to vesicle formation, but prior to vesicle consumption. This result is consistent with the interpretation that the Ypt1-D124N mutant protein interferes with wild type Ypt1p function.

XTP, But Not GTP, Relieves the Inhibitory Effects of Ypt1-D124N Protein

The *YPT1-D124N* mutation is analogous to a well-characterized mutation of EF-Tu. This mutation, EF-Tu-D138N, has been shown to change the nucleotide specificity of the protein from GTP to XTP (Hwang and Miller, 1987). The mutant protein binds and hydrolyzes XTP and performs its normal function in protein translation (Weijland and Parmeggiani, 1993). This change in nucleotide specificity has also been shown for the analogous mutation in *ras* (McCormick et al., 1987). In addition, it has been shown that exchange factors for EF-Tu and ras have higher affinity for the nucleotide-free mutant protein than for the wild type protein containing either GTP or GDP (Hwang and Miller, 1985; Lai et al., 1993; Haney and Broach, 1994). Based on these two findings, we reasoned that if Ypt1p-D124N, like EF-Tu-D138N, can bind XTP, the inhibition of stimulated guanine nucleotide exchange by Ypt1-D124N might be relieved by allowing the mutant protein to bind this nucleotide. Thus, binding of XTP by Ypt1p-D124N would reduce the affinity of the exchange factor for the mutant protein and would therefore reduce its effectiveness as an inhibitor in the exchange reaction. By this reasoning, the reversal of inhibition should be specific to XTP; GTP would be ineffective because the mutant protein cannot bind it. Moreover, if inhibition of the exchange activity for wild type Ypt1p is the reason for the inhibitory effect of the mutant protein on protein transport in vitro, XTP should also alleviate the transport block. In contrast, inhibition of exchange and transport by the other interfering mutant protein, Ypt1-N121I, should not be relieved by the addition of XTP because that mutation is not expected to promote an interaction with XTP.

To test these predictions, guanine nucleotide exchange assays were performed in the presence of Ypt1p-D124N and Ypt1p-N121I that had been preincubated with either GTP or XTP. Addition of XTP, but not GTP, relieved the inhibition of nucleotide exchange by Ypt1p-D124N (Fig. 5). Up to 80% of the inhibition caused by Ypt1p-D124N was reversed by addition of 200 μM XTP, while reversal by GTP was about 10-fold lower. The weak effect seen with GTP may be due to some binding at these high nucleotide concentrations, which were not achievable in the direct nucleotide binding assays shown in Table I. Relief of inhibition by XTP was specific to Ypt1p-D124N; neither XTP nor GTP significantly affected the inhibition of exchange by Ypt1p-N121I (Fig. 5).

To determine whether the inhibitory effects of Ypt1p-D124N on nucleotide exchange and protein transport are related, we assessed the ability of XTP to reverse the inhibition of the in vitro ER to Golgi transport reaction caused by Ypt1p-D124N. Increasing concentrations of XTP progressively reduced the inhibition of ER to Golgi transport caused by Ypt1p-D124N. We used a concentration of dominant mutant protein that elicited about 50% transport inhibition. Addition of 250 μM XTP restored transport to uninhibited levels. GTP was approximately fivefold less effective than XTP. As expected, neither nucleotide significantly alleviated the block caused by the Ypt1p-N121I protein (Fig. 6). Similar results were obtained in in vitro transport experiments using cytosol from yeast cells expressing the dominant mutant Ypt1 proteins.

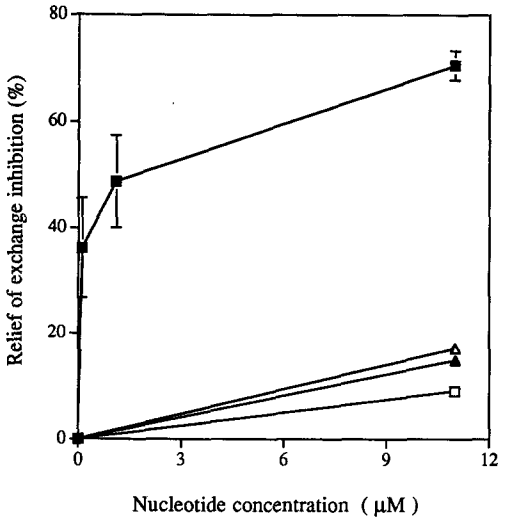

Figure 5. XTP alleviates the inhibition of stimulated guanine nucleotide exchange by Ypt1-D124N protein. Ypt1-D124N (*squares*) or Ypt1-N121I (*triangles*) proteins (1.55 μM) were preincubated with varying concentrations of nucleotide, XTP (*filled symbols*) or GTP (*empty symbols*), in the presence of 5 mM EDTA for 5 min at 30°C, and then added to a final concentration of 0.09 μM to a GDP release assay as described for Fig. 2 *a*, except that incubation was carried for 60 min. At this time percent stimulated exchange is 47.2 in the absence of inhibitory protein; and 17.7 in the presence of inhibitory protein. Results are presented as relief of stimulated exchange inhibition by nucleotides; nucleotide concentration is the concentration present in the exchange assay. Data shown are averages of two experiments and are representative of at least three independent experiments.

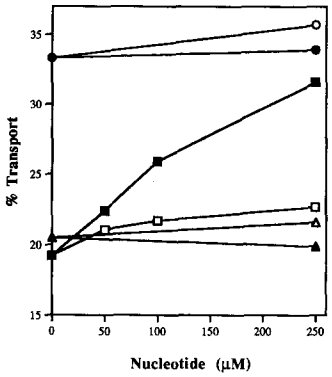

Figure 6. XTP alleviates the inhibition of in vitro transport caused by Ypt1-D124N protein. Ypt1-D124N inhibition of ER to Golgi in vitro transport is relieved by XTP (*filled symbols*) but not by GTP (*empty symbols*). Ypt1-D124N (*squares*) or Ypt1-N121I (*triangles*) mutant proteins, or wild-type (*circles*) (2.5 μM) were incubated with various concentrations of nucleotide for 10 min on ice prior to addition to the in vitro transport mixture as described in Fig. 3. Final concentration of Ypt1p in the assay mixture was 0.8 μM. Data shown are averages of two experiments and are representative of at least three independent experiments using different sets of fractions. There are several possible reasons for the lower concentration of XTP needed for the relief of exchange inhibition (Fig. 5) versus in vitro transport inhibition (Fig. 6): First, the concentration of mutant proteins used in the exchange inhibition experiment was about 10-fold lower than that used in the in vitro transport experiment (0.09 and 0.8 μM, respectively). Second, the preloading of the Ypt1 proteins with XTP for the exchange experiment was done in the presence of EDTA at 30°C, for more efficient loading. EDTA was not used in the transport experiments, since it inhibits this reaction, and the preloading was done on ice. Third, in the GDP release assay the concentration of nucleotides, which might protect the added XTP from degradation, was 75-fold higher than that included in the in vitro transport reaction (1.5 mM and 20 μM, respectively).

In these experiments both mutant cytosols inhibited transport, but only the cytosol from cells expressing Ypt1p-D124N could promote transport in the presence of XTP (data not shown). Thus, the dominant mutant proteins expressed in yeast cells behave in the same way as the proteins expressed in bacteria. The ability of XTP to relieve inhibition of both guanine nucleotide exchange and vesicular transport by Ypt1-D124N protein indicates that the nucleotide-free form of the mutant protein is the active inhibitor. It further suggests that inhibition of ER to Golgi transport by the dominant mutant proteins is due to inhibition of the guanine-nucleotide exchange factor that acts on Ypt1p.

Discussion

In this paper we provide strong support for an essential role of an exchange factor that acts on a rab-type GTPase in protein transport. It had previously been shown that *DSS4*, the only known yeast gene encoding an exchange factor for a rab protein, is not essential for viability. Moreover, its function does not seem to be needed for cell growth or secretion (Moya et al., 1993). This observation was surprising because at least one of the rab proteins that Dss4 regulates, Sec4p, is essential for viability and secretion (Salminen and Novick, 1987), and it has left open the question of whether nucleotide exchange factors for rab-type GTPases play an essential role. Injection of Mss4p into the squid giant nerve terminal facilitates neurotransmitter release, suggesting a role for this exchange factor for rab proteins in vesicular transport (Burton et al., 1994). Our results strongly suggest that the nucleotide exchange factor for Ypt1p is essential for its function in vesicular transport. Thus, purified dominant mutant Ypt1 proteins, N121I and D124N, inhibit both stimulated nucleotide exchange for wild type Ypt1p and in vitro ER to Golgi protein transport. Inhibition of both stimulated exchange and the transport reactions exhibit similar characteristics: similar amounts of the mutant Ypt1 proteins are required, and inhibition by Ypt1p-D124N can be relieved by the addition of XTP. We have evidence to suggest that the dominant mutant proteins do not block other known accessory proteins that regulate Ypt1p function, such as a GTPase-activating protein (GAP) or a GDI. Our recent results demonstrate that the dominant mutant proteins do not inhibit a GAP activity that acts on Ypt1p (Jones, S., and N. Segev, unpublished data). GDI is probably not the target for these mutant proteins since it has been shown to interact exclusively with the prenylated form of rab proteins (Araki et al., 1990; Regazzi et al., 1992; Soldati et al., 1993), and we found that unprenylated Ypt1 mutant proteins act in a dominant inhibitory fashion in vitro and in vivo. While it is still a formal possibility that an undescribed Ypt1-interacting protein that is essential for protein transport is also a target for the dominant mutant proteins, evidence from two other GTPases supports the interpretation that the exchange factor is the only target for these nucleotide-free dominant mutant proteins. The dominant effect of mutations in yeast *RAS2* and bacterial

EF-Tu, which render proteins that are defective in guanine nucleotide binding and that sequester their cognate exchange factors, can be suppressed in vivo by over-expression of the gene for the relevant exchange factor. Thus, *RAS2-G22A* can be suppressed by over-expression of Cdc25p, the exchange factor for Ras2p, and EF-Tu-K136E/Q can be suppressed by over-expression of EF-Ts, the exchange factor for EF-Tu (Powers et al., 1989; Hwang et al., 1989, respectively). The conservation between Ypt1, ras and EF-Tu with regard to the behavior of these mutants (defects in nucleotide binding, inhibition of the nucleotide exchange factor and the change in nucleotide specificity of the D124N mutant protein from GTP to XTP) strongly suggests that other aspects of their function will also be conserved. In addition, except for *DSS4*, exchange factors for GTPases that are essential for viability are themselves essential: e.g., *CDC25* (see above) (Broek et al., 1987; Robinson et al., 1987), and *SEC12*, the exchange factor for *SAR1*, which encodes a GTPase that is involved in the vesicle budding step of protein transport (Nakano et al., 1988; Barlowe and Schekman, 1993). *DSS4* might be exceptional either because there is a second nucleotide exchange factor for Sec4p, or because Sec4p function is less dependent on such a factor, as it has been shown to possess a high rate of intrinsic nucleotide exchange (Moya et al., 1993). The conclusion about the essential role of the exchange factor that acts on Ypt1p must now be supported by genetic and biochemical analyses after the gene has been identified.

The mechanism by which mutations in the NKXD consensus domain of rab proteins exert their dominance is not known. Our results suggest that the Ypt1-N121I and Ypt1-D124N mutant proteins exert their dominant effects by interfering with the wild type Ypt1p function, and that this interference is achieved by the sequestration of the exchange factor for Ypt1p by the nucleotide-free mutant protein. This model has been suggested for dominant interfering ras and EF-Tu mutations in the NKXD domain (see above). Our data support the generalization of this mode of dominance to rab-type GTPases. The suggestion that the dominant mutant Ypt1 proteins are nucleotide-free is supported by two pieces of evidence. First, we could not detect binding of GDP or GTP by these proteins. Second, the inhibition caused by the Ypt1p-D124N could be relieved by the addition of XTP, the nucleotide that it is predicted to bind. The further suggestion that the dominant effect of these proteins on protein transport is exerted by interference with the interaction of the wild type Ypt1p and its exchange factor is supported by two facts. First, inhibition of both the stimulated exchange and the transport reactions show similar characteristics (see above). Second, the block of transport caused by the dominant protein is identical to the block caused by loss of function of Ypt1p (Rexach and Schekman, 1991; Segev, 1991), in a step that lies between vesicle budding and vesicle fusion with the target membrane. The sequestration of the exchange factor by a dominant interfering mutant rab protein, makes such mutations valuable tools for the genetic or biochemical isolation of exchange factors for rab-type GTPases.

YPT1-D124N is a novel dominant mutation of *YPT1*. This mutation is analogous to the EF-Tu-D138N mutation, which has been shown to alter the nucleotide specificity of that protein from GTP to XTP (Hwang and Miller, 1987). Weijland and Parmeggiani (1993) demonstrated that binding of XTP to the mutant protein allowed it to perform its normal function. Likewise, the analogous mutation in *H-ras*, D119N, was demonstrated to have little or no effect on its interaction with exchange factors, GAP, and Raf (Zhong et al., 1995). The data presented above strongly suggest that a similar change in nucleotide specificity is caused by the *YPT1-D124N* mutation. Thus, both of the inhibitory effects caused by this mutant protein can be alleviated by the addition of XTP. Since this similarity exists between proteins as distant in function and primary structure (outside the GTP-binding consensus domains) as EF-Tu, ras, adenylosuccinate synthetase and Ypt1 (Hwang and Miller, 1987; McCormick et al., 1987; Kang et al., 1994; and this study, respectively) we predict that the analogous mutation will also cause the change in specificity from GTP to XTP in other rab proteins. Such mutations can be used for functional analysis of yeast and mammalian rab-type GTPases in a conditional manner, since the dominant inhibitory effect is reversed by adding XTP. Most importantly, such mutations can be used to follow an individual GTPase in crude extracts, since the mutant protein will be the only "GTPase" in the cell extract that can utilize XTP (Weijland and Parmeggiani, 1993).

There are several open questions regarding the function of exchange factors for rab proteins. One question is whether exchange factors have specific rab targets. This question is especially important since dominant mutations analogous to the *YPT1-N121I* have been used for the elucidation of specific steps at which different rab proteins function in vivo and in vitro (Gorvel et al., 1991; Bucci et al., 1992; Tisdale et al., 1992; Wilson et al., 1994). Since these mutations probably exert their inhibition by sequestering exchange factors, such experiments will not be conclusive until the specificity of the relevant exchange factor has been determined. Yeast Dss4p and mammalian Mss4 can stimulate release of GDP in vitro from more than one rab, i.e., not only from Sec4, but also to a lesser extent from Ypt1 and Rab3A (Burton et al., 1993; Moya et al., 1993). Mss4 was shown to interact with a subset of the rab GTPase family that is involved in the exocytic pathway, including the Ypt1p mammalian functional homologue, Rab1 (Burton et al., 1994). It is not clear whether this lack of specificity also exists in vivo. On the other hand, the exchange activity that we have identified appears to act preferentially on Ypt1p in vitro; the new factor, present in a crude cellular fraction, does not stimulate nucleotide exchange for Sec4p, which is the closest known family member of Ypt1p in yeast cells (Jones, S., R. J. Litt, and N. Segev, manuscript in preparation). In addition, the Ypt1p-N121I mutant, which inhibits the exchange activity for Ypt1p, does not seem to perturb Sec4p function in vivo. This mutation elicits blocks only in the early secretory steps that are dependent on Ypt1p function (Jedd, G., C. J. Richardson, R. J. Litt, and N. Segev, manuscript in preparation). However, we cannot rule out the possibility that the exchange factor that we have identified can stimulate nucleotide exchange by other rab GTPases that function in the yeast exocytic pathway. Another question concerns the mechanism of action of the exchange factors for rab

proteins. They might be needed exclusively for regulation of the cycling of rab proteins from the GDP- to the GTP-bound form. In that case, the incomplete block of both the in vivo and in vitro transport elicited by the dominant mutant Ypt1 proteins might be explained by the unstimulated exchange of nucleotide bound to the wild type Yptlp. Alternatively, exchange factors for rab proteins could have additional functions such as recruitment of rab proteins to membranes (Dirac-Svejstrup et al., 1994; Soldati et al., 1994; Ullrich et al., 1994), promoting interaction of rab proteins with other proteins during complex formation (Lian et al., 1994; Søgarrd et al., 1994), or serve as targets for regulation by other enzymatic systems. The latter function was suggested for the GTPase cascade in yeast bud formation, which involves regulation of an exchange factor for one GTPase by another GTPase (Chant, 1994). Isolating the genes that encode exchange factors for the different rab proteins and determining the subcellular localization of their protein products should help resolve these issues.

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